Development and validation of an UHPLC-MS/MS method for β2-Agonists in human urine and application to real samples

Manuscript Details

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Title	Development and validation of an UHPLC-MS/MS method for β2-agonists quantification in human urine and application to clinical samples
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Abstract

A fast analytical method for the simultaneous detection of 24 β2-agonists in human urine was developed and validated. The method covers the therapeutic drugs most commonly administered, but also potentially abused β2-agonists. The procedure is based on enzymatic deconjugation with β-glucuronidase followed by SPE clean up using mixed-phase cartridges with both ion-exchange and lipophilic properties. Instrumental analysis conducted by UHPLC–MS/MS allowed high peak resolution and rapid chromatographic separation, with reduced time and costs. The method was fully validated according ISO 17025:2005 principles. The following parameters were determined for each analyte: specificity, selectivity, linearity, limit of detection, limit of quantification, precision, accuracy, matrix effect, recovery and carry-over. The method was tested on real samples obtained from patients subjected to clinical treatment under chronic or acute therapy with either formoterol, indacaterol, salbutamol, or salmeterol. The drugs were administered using pressurized metered dose inhalers. All β2-agonists administered to the patients were detected in the real samples. The method proved adequate to accurately measure the concentration of these analytes in the real samples. The observed analytical data are discussed with reference to the administered dose and the duration of the therapy.

Keywords	β2-agonists; UHPLC; MS/MS; human urine; validation; asthma
Taxonomy	Determination of Drugs in Biological Samples, High-performance Liquid Chromatography With Sub-3-micron Particle
Manuscript category	Bioanalytical Applications

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Orbassano, November 20th , 2017

Editorial Board
Journal of Pharmaceutical and Biomedical Analysis

Dear Editors,
please find enclosed the revised version of the manuscript entitled "Development and validation of an UHPLC-MS/MS method for β2-agonists detection in human urine and application to real samples".

The authors are: Cristina Bozzolino, Marta Leporati, Federica Gani, Cinzia Ferrero, Marco Vincenti.

Moderate revision and modification of the article in light of the reviewer comments was requested.
All the comments made by the Reviewers have been addressed and all the requested changes were made in the text. An itemized list of these responses and changes is reported in a dedicated file.

Thank you for reconsidering the paper for publication in Journal of Pharmaceutical and Biomedical Analysis.

Best regards.

Yours faithfully,

Marta Leporati

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COMMENTS FROM THE EDITORS AND REVIEWERS

REVIEWER 1

Comments to Author

This manuscript describes the validation UPLC-MS/MS method for the quantitation of 24 β2-agonists in urine. The authors selected a large group of β2-agonists for its determination and quantitation in samples. In these regards, the work is a development in analytical methodology with potential applications in therapeutic monitoring and forensic toxicology.

The work is well written and easy to follow. The validation of the method is very complete and well performed. I am glad to see that the LOD and LOQ values were experimentally confirmed (line 149). Moreover, some interesting conclusions arise from the analysis of clinical samples.

Specific comments:

- The research group has experience in the determination and quantitation of β2-agonists with GC-MS (ref. 23 in the text). The advantages of the present method, compared to the previous one, are justified by the observed improvement in sensitivity, which is of particular importance for some analytes. This must be due to the use of tandem MS, although this is not specified in the text. The use of LC instead of GC is justified by the growing implementation of LC for the study of β2-agonists, since this technique does not require previous derivatization steps. Although these ideas are spread along the manuscripts, a deeper discussion of the advantages of this method compared to other commonly used methods would be very useful for the reader. This could be done in an additional section between current sections 3.2 and 3.3. In this section, specific points such as the time for sample preparation, use of reagents, sensitivity or selectivity could be discussed. A new 3.3 section was included, as suggested.

- The reasons for the selection of urine as sample for the determination of β2-agonists is not stated in the manuscript. This could also be useful for readers. A brief discussion on this topic was introduced in lines 67-71.

Other minor comments:

- Title: I suggest to replace the term “real samples” by “clinical samples” in the title. The change was made.

- Graphical abstract: If the authors are determined to submit a graphical abstract, the file size should be proportional to 531 × 1328 pixels. Please, have a look at some examples here: https://www.elsevier.com/authors/journal-authors/graphical-abstract The graphical abstract was reviewed.

- Highlights: Please, highlight that the method has been validated according to ISO 17025:2005 principles. This is the main point in this work. A new highlight was added.

- Abbreviations: Avoid defining abbreviations that are standard in the journal: https://www.elsevier.com/__data/promis_misc/JPBA%20Abbreviations.pdf Many defining abbreviations were removed (lines 34, 68, 73, 74, 76, 77, 87, 153, 155, 162, 186, 187, 193, 293).
- Line 65. “These methods are…” The change was made.
- Line 162. I guess these individuals were not in any treatment with β2-agonists. Please, specify what the term "blank" means. The text “not in therapy with β2-agonists” was added.
- Line 186. “With respect to the RSD” The change was made.

REVIEWER 2

The manuscript deals with the development of a development of an UHPLC-MS/MS method for beta2-agonists determination in human urine samples.

The paper is well written and organized. The validation procedure is precisely carried out and the topic of the paper is worthy of publication in the JPBA after some revisions of the text.

Comments:

-Title: beta2-agonists were quantified in urine human samples and not only detected. I suggest to change the title in turn. The change was made.

- L134-136. I don't understand why the eluate was reconstituted with a mixture 95/5 metanol/water, instead of a mixture 95/5 water/methanol, i.e. the initial gradient composition of the mobile phase. This is enough strange. Yes, it was wrong. It was a typographical error. The right percentage was replaced.

- L141. Modify "BEH C18 column (2.1 mm x 100 mm, 1.8 um)" and specify the geometry of the pre-column too. The change was made.

-L146. delete "Sciex", since already afterward reported. The change was made.

- L 264. Did the authors use weighting factors for calibration plots? No, we did not.

- L359, 382 Add the standard deviation. Media value and standard deviation was added. In L359 also a typographical error was correct: as reported in Table 5 for patients under acute therapy with formoterol, all the detected concentrations were lower than 2.0 ng/mL and not 0.70 ng/mL.

- L 374, 379. Change "ppb" in appropriate concentration unit. The change was made.

- Table 1. Column 6. Change "target fragment" with " Quantifier fragment" for analogy with column 9. The change was made.

- Table 2. The linearity range reported in this table are not correct. The lowest value of the linearity range must be always the LOQ value! The change was made.

- Table 2. In this table, the LOD values are the same of LOQ values for 3 analytes (clencicloexerol, hydroxymethylclebuterol and salmeterol). Although the authors use the Hubaux-Vox method for the

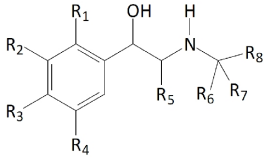
calculation of LODs and LOQs, I ask them to justify these values, as the meaning of LOD and LOQ are different, and if LOD = LOQ an explanation in the text must be given. LOD and LOQ appeared to be the same for these analytes because of the approximation to a single significant digit. By adding a decimal digit, as in the new Table 2, different and more correct expression of LOD and LOQ is obtained.

- Caption of figure 3. Please check the significant digits and add the standard deviations. Significant digits were checked and standard deviations were added.

- Figures 2 and 3. Show both the quantifier and qualifier transitions for each chromatographic peak. The qualifier fragments were added in both figures. The text “In black is reported the quantifier fragment and in red the qualifier fragment.” was added in the figure captions.

Highlights

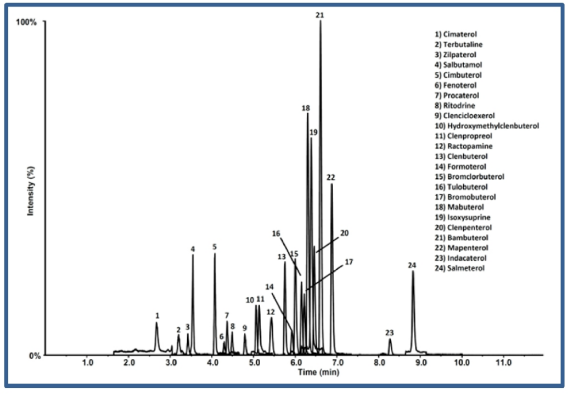
- A UHPLC-MS/MS method was developed for the detection of 24 β_2-agonists in urine
- The method was fully validated according to UNI EN ISO IEC 17025:2005
- The suitability of the method was proved by real samples testing
- The β_2-agonists urinary concentration varies with administered dose and duration



24 β_2-agonists

Human urine

UHPLC-MS/MS



Validated according to
UNI EN ISO IEC 17025:2005

Salmeterol

Salbutamol

Formoterol

Indacaterol

Real samples from clinical treatments

DEVELOPMENT AND VALIDATION OF AN UHPLC-MS/MS METHOD FOR β_2-AGONISTS ~~DETECTION~~ QUANTIFICATION IN HUMAN URINE AND APPLICATION TO ~~REAL~~ CLINICAL SAMPLES

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KEYWORDS: β_2-agonists; UHPLC; MS/MS; human urine; validation; asthma.

ABSTRACT

A fast analytical method for the simultaneous detection of 24 β_2-agonists in human urine was developed and validated. The method covers the therapeutic drugs most commonly administered, but also potentially abused β_2-agonists. The procedure is based on enzymatic deconjugation with β-glucuronidase followed by SPE clean up using mixed-phase cartridges with both ion-exchange and lipophilic properties. Instrumental analysis conducted by ~~ultra-high performance liquid chromatography–tandem mass spectrometry (~~UHPLC–MS/MS~~)~~ allowed high peak resolution and rapid chromatographic separation, with reduced time and costs. The method was fully validated according ISO 17025:2005 principles. The following parameters were determined for each analyte: specificity, selectivity, linearity, limit of detection, limit of quantification, precision, accuracy, matrix effect, recovery and carry-over. The method was tested on real samples obtained from patients subjected to clinical treatment under chronic or acute therapy with either formoterol, indacaterol, salbutamol, or salmeterol. The drugs were administered using pressurized metered dose inhalers. All β_2-agonists administered to the patients were detected in the real samples. The method proved adequate to accurately measure the concentration of these analytes in the real samples. The observed analytical data are discussed with reference to the administered dose and the duration of the therapy.

1. Introduction

β-adrenergic agonists, or β_2-agonists, are among the oldest and most commonly prescribed therapeutic agents for the treatment of asthma, a chronic inflammatory airway disorder. β_2-agonists are widely used also to treat chronic obstructive pulmonary disease (COPD) and numerous other respiratory diseases, either alone or in combination with bronchodilators and corticosteroids [1]. From the chemical point of view, β_2-agonists are phenyl β_2-ethanolamines bearing different substituents on the amino- nitrogen and the phenylic ring, with chemical structures closely related to endogenous catecholamines [2].

Because of their therapeutic stimulatory effect on the breath capacity and central nervous system, β_2-agonists are occasionally abused by chronic asthmatic patients, possibly leading to severe intoxication. They are also misused in sport and animal husbandry as growth promoters, due to their side effects on protein synthesis and lipolysis, that depend on the dose and administration route, possibly resulting in an anabolic-like action [3]. For these reasons, the identification and quantification of β_2-agonists in various biological matrices is important, and requires selective and sensitive analytical methods.

Among the biological matrices, urine presents many advantages for drugs detection. Unlike blood, urine sampling is non-invasive and naturally produce a larger volume. In urine, β_2-agonists are typically excreted as either phase I or phase II metabolites, accompanied by a large percentage of the unmodified drug, depending on the dose and administration route. This allows to explore a wider temporal window after the last assumption, extending to a few days.

Laboratory methods for β_2-agonists are commonly based on a chromatographic technique coupled with ~~mass spectrometry (~~MS~~)~~, which undoubtedly offers great performances for complex mixture analysis, in terms of analytical sensitivity and specificity, detection limits and quantitation capabilities [4]. These method~~s~~ are required for confirmation purposes in official zootechnics controls [5], but they can also be used as screening methods, since they generally provide the required sensitivity for a wide range of analytes, together with qualitative and quantitative information [6]. ~~Gas chromatography-MS (~~GC-MS~~)~~ methods had been initially reported for β_2-agonist analysis [7,8], but they were subsequently replaced by LC-MS ~~liquid chromatography – mass spectrometry~~ methods ~~(LC-MS)~~ that require no derivatization steps, and allow higher sensitivity and specificity by means of MS/MS ~~tandem MS~~ conditions ~~(MS/MS)~~ [9,10].

Recent efforts were made to develop analytical methods based on ~~ultra-high performance liquid chromatography (~~UHPLC~~)~~-MS/MS to obtain high peak resolution, high sensitivity, rapid

chromatographic separation, and reduced analysis time and costs at the same time. To date, only few UHPLC methods have been described to detect simultaneously a large set for β_2-agonists. Multi-target methods were developed respectively for 18 β_2-agonists detection in bovine urine [11], 20 β_2-agonists in bovine hair [12], 16 β_2-agonists in pig liver, kidney and muscle [13], and 11 β_2-agonists in human urine [14]. The latter method reported homogeneous LOD values of 0.1 ng/mL for all targeted analytes, including salmeterol, whose actual concentration in urine after clinical administration is frequently below this 0.1 ng/mL limit [15].

To improve sensitivity toward β_2-agonists, several clean-up procedures have been proposed before instrumental detection. Most clean-up methods were based on ~~solid phase extraction (~~SPE~~)~~ using different sorbents [16,17], but also other techniques such as matrix solid phase dispersion [18], immunoaffinity based techniques [19] and supercritical fluids extraction [20] have been reported. Lately, molecularly imprinted polymer technology [21,22] has also been experimentally applied to the clean-up of β_2-agonists.

Until recently, our laboratory used a fast-GC-MS method for the simultaneous determination of 15 β_2-agonists in human urine [23]. The need to improve further the sample throughput and general applicability made us develop a new sensitive and robust UHPLC-MS/MS method for the detection of 24 β_2-agonists (Figure 1) in human urine, covering the therapeutic drugs most commonly administered, but also potentially abused β_2-agonists and "cocktails", i.e. mixtures of β_2-agonists with or without other anabolic substances [24]. The present method was validated in agreement with the UNI EN ISO IEC 17025:2005 [25] principles and successfully applied to the therapeutic monitoring of patients with respiratory-related diseases, after their treatment with various β_2-agonists.

2. Material and methods

2.1. Reagent and chemicals

Bambuterol hydrochloride, bromchlorbuterol hydrochloride, brombuterol hydrochloride, cimaterol, cimbuterol, clenbuterol hydrochloride, clecicloexerol hydrochloride, clenpenterol hydrochloride, clenproperol, fenoterol hydrobromide, formoterol fumarate dihydrate, hydroxymethylclenbuterol, isoxsuprine hydrochloride, mabuterol hydrochloride, mapenterol hydrochloride, procaterol hydrochloride, ractopamine hydrochloride, ritodrine hydrochloride, salbutamol, terbutaline sulfate, tulobuterol hydrochloride, methanol, formic acid (LC-MS Ultra grade, ~98%), ammonium acetate, β-glucuronidase type II from helix pomatia, and ammonium

hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Indacaterol and formoterol-13C-d_3 were purchased from AlsaChim (Illkirch Graffenstaden, France). Salmeterol xinafoate was supplied by the European Directorate for the Quality of Medicines & HealthCare (, Strasbourg, France). Zilpaterol was obtained from Spectra 2000 srl (Rome, Italy). Clenbuterol-d_6 was supplied by the Netherlands National Institute for Public Health and the Environment (RIVM, Bilthoven, Netherlands). Salbutamol-d_3 was purchased from LGC (Teddington, Middlesex, United Kingdom), salmeterol-d_3 from CDN Isotope (Pointe-Claire, Quebec, Canada). Hydrochloric acid (HCl) 37% was purchased from Carlo Erba (Milan, Italy). Ultrapure water was obtained using a Milli-Q® UF apparatus (Millipore, Bedford, MA, USA).

All stock standard solutions were prepared in methanol at 1 mg/mL and stored at –20° C in the dark until use. Working solutions were prepared by dilution with methanol.

Four deuterated internal standards (salbutamol-d_3, clenbuterol-d_6, formoterol-^{13}C-d_3, and salmeterol-d_3) were used for the quantitation procedure at equal concentration. Blank urine samples were collected from healthy volunteers (laboratory personnel), pooled, and used as the working matrix to develop and validate the analytical protocol.

2.2. Sample preparation

An aliquot (3 mL) of urine specimen was centrifuged at 3500 rpm for 5 min. Subsequently, 2.5 mL of supernatant was transferred into 30-mL glass tubes and 25 µL of the internal standard (IS) solution (concentration of 0.1 µg/mL) was added. A 0.2 M aqueous ammonium acetate solution at pH 5.0 was added (2.5 mL) into the samples, together with 10 µL of β-glucuronidase. Enzymatic deconjugation was carried out for 2 h at 37° C. The samples were allowed to cool down to room temperature and then loaded onto a SPE Strata-XC 33 µm, 60 mg x 3 mL cation exchange cartridge (Phenomenex, Castel Maggiore (BO), Italy), previously conditioned with 2 mL of methanol and 2 mL of ultrapure water. After sample loading, the SPE cartridges were washed with 2 mL HCl 0.1 M and then 2 mL of methanol. The target analytes were eluted with 2 mL of methanol doped with 5% (v/v) of ammonium hydroxide. The eluate was evaporated to dryness under a gentle stream of nitrogen and at 50° C using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK), and then reconstituted with 100 µL of a methanol-0.1% formic acid aqueous solution (~~9~~5:9̲5, v/v) and transferred into the analytical vial for UHPLC-MS/MS analysis.

2.3. UHPLC-MS/MS analysis

Chromatographic separations were performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Palo Alto, CA, USA), including a vacuum degasser, a binary pump, an autosampler and a column thermostat. The liquid chromatograph was equipped with a Waters (Milford, MA, USA) BEH C18 column ~~–~~ (2.1 mm x 100 mm x 1.~~8~~ 7 µm) ~~column~~ maintained at 40°C and a ~~n analogue~~Waters (Milford, MA, USA) BEH C18 vanguard pre-column (2.1 mm x 5 mm x 1.7 µm). The chromatographic run was carried out by a binary mobile phase of a 0.1% v/v aqueous formic acid solution and methanol, using the following program: isocratic with 5% methanol for 2 min; linear gradient from 5% to 80% in 8 min; isocratic with 80% methanol for 1 min; total run time 15 min. The injection volume was 1 µL and the flow-rate was 0.4 mL min^{-1}. The LC was interfaced to a ~~Sciex~~ QTRAP® 4500 triple–quadrupole mass spectrometer (Sciex, Ontario, Canada), operating in ~~electrospray ionization (~~ESI~~)~~ – positive ion mode. The other MS parameters were set as follows: curtain gas: 35 psi; nebulizer gas: 45 psi; heater gas: 40 psi; probe temperature: 550 °C; IS voltage: +3500 V. Ion acquisition was operated at unit mass resolution in the ~~selected reaction monitoring (~~SRM~~)~~ mode, using the two transitions from the protonated molecular ion of each analyte to the fragment ions indicated in Table 1.

2.4. Validation

The analytical method was validated in accordance with the criteria and recommendations of UNI EN ISO/IEC 17025:2005 international standard [25]. The following parameters were investigated: specificity, selectivity, linearity range, ~~detection and quantification limits (~~LOD and LOQ~~)~~, intra-assay precision and accuracy. Carry-over, recovery and matrix effect were also investigated. Blank human urine samples obtained from healthy volunteers were used for the validation experiments following the analytical protocol described above.

2.4.1. Specificity and Selectivity

Ten blank urine samples from different individuals not in therapy with β_2-agonists were analysed. The occurrence of possible interferences from endogenous substances was tested by monitoring the SRM chromatograms characteristic for each investigated compound at the expected retention time interval. The signal-to-noise ratio (S/N) was measured on the least intense mass transition at the expected retention time. The noise was measured from the end of the peak till 0.05 min after it. A S/N<3 was considered satisfactory in order to verify the method specificity.

Identification criteria for the analytes were established according to Decision 2002/657/EC [5]. For each analyte, one qualifying mass transition was monitored, in addition to the primary fragmentation (Table 1). Variations of relative peak intensities were tested at three concentration levels (concentration of first, third and fifth point of the calibration curves). Retention time (t_R) precision was also tested at the same concentrations.

2.4.2. Linearity, LOD and LOQ

The linear calibration model was checked by analyzing (five replicates) blank urine samples spiked with the working solution at six concentration levels (see Table 2). The calibration was completed by internal standardization. The linear calibration parameters were evaluated using the least squares regression method. Determination coefficient (R^2), slope, and intercept were calculated, and several significance tests were performed to evaluate linearity, including Lack-of-Fit test, ~~Analysis of Variance (~~ANOVA~~)~~ test, Mandel's test, evaluations of the ~~relative standard deviation of the slope (~~RSD slope test~~)~~ and the residual plots, together with the analysis of the deviation from back-calculated concentrations.

The tests were passed when the calculated values (F_{exp}) proved to lower than the corresponding critical value at α=0.05 significance level: F_{crit} = 2.776 (n_1 = 4 and n_2 = 24 degrees of freedom) for the Lack-of-Fit test, F_{tab} = 3.842 (n_1 = 1 and n_2 = 28 degrees of freedom) for the ANOVA test, and F_{crit} = 2.62 (n_1 = 5 and n_2 = 24 degrees of freedom) for the Mandel's test. With respect to the RSD~~DS~~ slope and back calculation tests, the adopted threshold values were 5.00% and 20%, respectively. The residual plot analysis turned out positive when a random pattern was observed, namely there was no recognizable trend of the residuals as a function of the concentrations.

The LOD values were estimated using the Hubaux and Vos approach [26]. Five independent calibration lines were prepared for all the target analytes and a significant level of 95% was selected at the corresponding number of degree of freedom. Then, LOD value was calculated by applying the Hubaux-Vos algorithms. LOQ was calculated as two times the LOD.

The calculated LOD and LOQ values were subsequently tested in experiments with blank urine samples spiked with analyte concentrations extremely close to the respective LOD and LOQ values, to confirm the estimation correctness.

2.4.3. Precision and accuracy

For all analytes, intra-day precision (expressed as percent variation coefficient, CV%) and accuracy (expressed as bias%) were evaluated by analyzing ten blank urine samples spiked with the working solutions at low, medium and high calibration level (the same concentrations used for selectivity evaluation). Intra-assay precision was considered satisfactory when CV% values were below 25% for the low calibration level and below 15% for the other levels. Satisfactory accuracy was achieved when the experimentally determined average concentration lied within ±15% from the expected value. All results are reported in Table 3.

2.4.4. Carry-over

Carry-over was evaluated by injecting an alternate sequence of five blank urine samples spiked with all the analytes at concentrations higher than 5 ng/mL and five blank urine samples. To ensure the absence of any carry-over effect, S/N ratios had to be lower than 3 for each monitored transition.

2.4.5. Matrix effect and extraction recovery

The matrix effect was evaluated as the percentage ratio between the area (mean value from five replicates) obtained by adding the analyte to the matrix extract and the one determined in a blank deionized water solution spiked with the analyte at the same concentration after the extraction step. The percentage difference highlighted matrix suppression (values below 100%) or enhancement (values above 100%) [27]. Matrix effect was estimated at the first level of the calibration curves.

The extraction recovery represents the percentage of analyte extracted after sample workup from a blank urine sample originally spiked with a known concentration of target analytes. It was calculated as the ratio between the analyte area determined in the extracted samples (5 replicates) and the one determined in blank samples (5 replicates) in which the analyte was added after the extraction step. Extraction recoveries were estimated at the three concentration levels cited above.

2.5. Real urine samples

In order to verify the complete analytical procedure on authentic specimens, real urine samples were collected from 60 compliant patients, who were subjected to pharmacological treatment with β_2-agonists at San Luigi Gonzaga University Hospital. General information on the patients and

their treatment with β_2-agonists is listed in Tables 4 and 5. Briefly, β_2-agonists were administered using pressurized metered dose inhalers. The active ingredients were salbutamol (100 µg/puff), salbutamol/beclometasone (100 µg/puff), salmeterol/fluticasone (25 or 50 µg/puff), formoterol/budesonide (4.5 or 9 µg/puff), formoterol/beclometasone (6 µg/puff) and indacaterol (150 µg/puff). All analyses were routinary therapeutic controls executed for clinical purposes. However, the patients provided written informed consent before attending the study, and an anonymous code was attributed to each subject participating to the present study to respect privacy regulations.

3. Results and discussion

3.1. Method development

The optimized UHPLC-MS/MS method allowed the simultaneous determination of 24 β_2-agonists in human urine. The whole chromatographic run, comprehensive of the time required for column re-equilibration before the following injection, was completed in less than 15 min. Retention times ranged between 2.70 min (cimaterol) and 8.84 min (salmeterol). Figure 2 shows the SRM chromatograms recorded from a blank urine spiked with all the analytes at 0.5 ng/mL.

The choice of the SPE procedure, that involved mixed-phase cartridges with both ion-exchange and lipophilic properties, was made after comparison of literature results. In particular, a very exhaustive comparative study of various sorbents types was made by Dos Ramos et al on bovine urine as the matrix, suggesting the use of mixed-phase sorbent as the one yielding the best results in β_2-agonists recovery [28]. The present results confirm that optimal clean-up is observed by using mixed-phase SPE cartridges.

3.2. Validation results

3.2.1. Specificity and Selectivity

The SRM chromatographic profiles obtained from blank urine samples collected from 10 untreated subjects did not show the presence of any significant signal (S/N<3) at the relative retention time typical of all the studied compounds and ISs, indicating that the method is selective and no interfering substance is present in the biological matrices.

The analytes were clearly identified in all the spiked samples, according to the criteria reported in the Decision 2002/657/EC, that were fully satisfied.

3.2.2. *Linearity, LOD and LOQ*

The linear matrix-matched calibration model was checked by analyzing five replicate blank urine samples spiked with the working solutions at six final concentrations. More in detail, two intervals were investigated for the analytes listed in Table 2, depending on the typical therapeutic dose of each β_2-agonist and its expected concentration in urine: 0.03–2.5 ng/mL (0.03, 0.1, 0.25, 0.5, 1.0 and 2.5 ng/mL) or 0.15–5.0 ng/mL (0.15, 0.25, 0.5, 1.0, 2.5 and 5.0 ng/mL). Quantitative data resulting from area counts were corrected using the respective IS signal areas. All the linear calibration parameters turned out adequate and the results, reported in Table 2, confirm the appropriateness of the linear model. Further confirmation of the calibrations linearity was obtained from the analysis of the residual plots, since a random residuals pattern along the concentration range was observed for all the analytes.

LODs were calculated by the Hubaux-Vos technique [26] and the experimental verification tests confirmed the correct estimation of LOD and LOQ. The Hubaux-Vos method is founded on rigorous statistical basis and should be preferred with respect to the LOD and LOQ estimation made from the S/N values of blank and spiked samples or the average signal and its standard deviation. In particular, the latter methods do not define the baseline position and amplitude from which the S/N value should be calculated, introducing a potential factor of bias related to the operator's discretion. The LODs and LOQs values reported in Table 2 proved to be at least one order of magnitude lower than those recorded in our previous fast-GC-MS method [23]. This increase in sensitivity turned out to be particularly important for some analytes, i.e. salmeterol, commonly excreted at low concentration after administration.

3.2.3. *Precision and accuracy*

Intra-day data on precision and accuracy are reported in Table 3. The results show satisfactory repeatability, as the ~~percent variation coefficient (~~CV%~~)~~ is lower than 15% for all the spiked analytes at low, medium and high concentrations, with only five exceptions (16% for cimaterol, 19% for ritodrine, 16% for ractopamine, 17% for mapenterol and 18% for salmeterol at the lowest calibration level). The accuracy is also satisfactory, as the percent bias (bias%) ranges from -4.7% to +15%. Overall, all the experimental figures-of-merit satisfy the predetermined criteria (see paragraph 2.4.3).

3.2.4. *Carry-over*

The background chromatographic profiles of the main SRM transitions for each analyte, monitored during the analysis of blank urine injected after highly spiked samples, did not show the presence of any significant signal (i.e. the S/N value was always <3) at the retention times expected for the tested analytes, with the notable exception of procaterol that showed some carry-over effect in two runs out of five. It is recommended to inject a blank sample after the injection of the last point of the calibration curve and in general after any sample with high procaterol concentration.

3.2.5. Matrix effect and recovery

Recovery and matrix effect values are given in Table 3. In general, the results show satisfactory recovery values. The average extraction recovery is 100%: the minimum observed value is 80% for mapenterol at 0.03 ng/mL, while the maximum value equals 116% for fenoterol at 0.15 ng/mL. The average matrix effect is estimated around +1.2%: the highest negative effect is -33% for cimaterol, while the largest positive effect is +23% for clenbuterol. In conclusion, good extraction recoveries combined with acceptable matrix effect allowed the correct determination of all the target analytes.

3.3. Method improvement

The present UHPLS-MS/MS method was developed to be used in the daily laboratory activity in place of the fast-GC-MS procedure previously employed [23]. The former method provided reasonable sensitivity and adequate instrumental processing time to meet the requirements (in terms of LOQ and sample throughput) of most routine determinations. However, it needed to be updated to include the newest β_2-adreneregic receptor agonists, among which indacaterol, zilpaterol, isoxsuprine, and mabuterol, occasionally adopted in the clinical and veterinary practice or potentially abused. Moreover, the GC-MS method required a strong derivatization step under strictly controlled conditions, to reduce the polarity of most targeted analytes. For example, the derivatization with trimethylsilyltrifluoroacetamide introduced three TMS groups in salbutamol and formoterol and four TMS groups in salmeterol.

The new method is equally fast, does not require previous derivatization steps, and is validated for as many as 24 target β_2-agonists, namely all those potentially requested to our laboratory for both clinical and doping purposes. The combination of electrospray ionization with tandem mass spectrometry, together with the addition of SPE purification of the extract, considerably improved the sensitivity with respect to the previous method [23]. On average, LOD values were decreased by 1-2 orders of magnitude for all the analytes included in both methods. Moreover, a few specificity problems observed in the GC-MS method had been eliminated. For example, the signal obtained from low formoterol concentrations in real samples

was barely detectable in the GC-SIM profile as a shoulder at the right side of an interfering peak, making the quantification highly problematic. In the present method, the use of SPE purification and MS/MS detection removed all interferences from SRM profiles, enhancing both specificity and sensitivity.

~~3.3.~~3.4. *Real samples results*

All four β_2-agonists (formoterol, indacaterol, salbutamol and salmeterol) administered to the patients were detected in the real samples. A representative SRM profile for each analyte is reported in Figure 3, showing optimal chromatographic profiles at both high and low concentrations. The therapeutic, chronological, and analytical data for real clinical samples are reported in Table 4 (chronic therapy) and Table 5 (acute therapy).

~~3.3.1.~~3.4.1. *Indacaterol*

Indacaterol is a novel, long-acting inhaled β_2-adreneregic receptor agonist intended for long-term, single daily dose, maintenance treatment in patients with COPD [29]. The long-lasting pharmacological activity of indacaterol is due to the presence of a long, lipophilic side-chain in the chemical structure that binds to an exo-site on adrenergic receptors [30]. The urine of a 66 years old woman was collected 24 hours after the last assumption of 150 µg of indacaterol and two weeks long therapy. The patient had been in therapy with salbutamol for 16 years, as needed, but with poor control of the disease. Then, salbutamol was substituted with indacaterol, which required single daily dose. The drug was found in urine at the concentration of 3.93 ng/mL (see Figure 3, line C). Salbutamol was still detected at the concentration of 42.1 ng/mL, 18 days after its replacement (reported below).

~~3.3.2.~~3.4.2. *Salmeterol*

Salmeterol is a long-acting selective β_2-agonist used to control asthma in combination with inhaled steroid therapy. In contrast, salmeterol could be used as a monotherapy in COPD. Its concentration in urine after administration is very low, inasmuch as it is readily metabolized to α-hydroxysalmeterol [31]. The urine samples from twelve patients in chronic therapy with variable doses and collecting intervals after administration were analyzed. The results, reported in Table 4, show that higher average salmeterol concentration is detected from patients with longer therapeutic periods (more than 6 months), possibly because of some accumulation effect. For example, different salmeterol concentrations were detected from patients 012, 039 and 043, i.e. women of a similar age under the same therapy conditions and sampling interval. The

concentration obviously depends also on the delay with which urine has been collected after the last administration.

~~3.3.3.~~3.4.3. *Formoterol*

Formoterol is a potent long-acting β-agonist, typically applied by means of a metered dose inhaler. It shows rapid action offset, which is exploited in asthma - in association with inhaled steroid - not only for disease control but also as a reliever. In COPD, formoterol can be used in monotherapy. At high doses, it may also act as an anabolic agent, increase the heart rate, and produce excitement. Hence, formoterol might potentially be misused in sports for its stimulatory effect and possible anabolic action, although most studies on the effects of inhaled β-agonists did not show any improvement in the elite athletes performance [32].

Urine samples from patients who used formoterol in both acute and chronic therapy were analyzed. In chronic patients, the detected urinary concentrations of formoterol were scattered, varying in the range between fractions and units of ppb, with two notable exception of higher levels (patients 030 and 046). According to the data reported in literature, formoterol is excreted in urine either as phase I (O-demethylation of the methoxyphenyl group and deformylation) or phase II metabolites (glucuronidation mainly at the phenolic position, but a benzyl glucuronide is also formed) [33], accompanied by a large percentage of unmodified drug [34]. No differences were observed between patients in therapy from more or less than sixth months and no clear dependence from the dosage was detected.

In patients under acute therapy, all the detected concentrations were lower than ~~0.70~~2.0 ng/mL (0.50±0.07 ng/mL). Urine samples collected too early (patient 015) or too late (patients 001-003-049) with respect to the administration lead to low or undetectable drug levels, although in patient 036 formoterol was still detected 48 hour after administration.

~~3.3.4.~~3.4.4. *Salbutamol*

Salbutamol is a widely prescribed β_2-agonist for relieving bronchospasm in patients with asthma and COPD [35]. The list of prohibited substances in sports published by the WADA specifies that the use of salbutamol is only permitted by inhalation. Administration by the oral or parenteral route or the administration of very large inhaled doses are forbidden due to an strong adrenergic stimulatory effect and an anabolic-like effect. In contrast, administration of therapeutic inhaled doses have no ergogenic effect [36].

The urine samples of 10 patients to whom salbutamol was administered in acute therapy were analyzed (see Table 5). Although relatively high concentrations of salbutamol were always measured, it is evident that the urinary concentration of salbutamol decreased from hundreds to tens of ~~ppb~~ ng/mL after 12 or more hours from the assumption (patients 026 and 027). The single recorded exception (patient 008) showing low salbutamol urinary concentration, actually received a small dosage of salbutamol. The persistence and possible accumulation of the drug in the body was supported by the analysis of an urine sample from a patient chronically treated with salbutamol (50+50 µg/day) for the last 16 years: 18 days after the therapy suspension, salbutamol was still present at the relatively high concentration of 42 ~~ppb~~ ng/mL (Patient 006 in Table 4). Less pronounced effect was recorded for a 38-years old male subject, who was in therapy since one year with 200 µg/day salbutamol: after 3 hours from administration, its urinary concentration was 57±7 ng/mL.

4. Conclusions

The UHPLC-MS/MS method developed and validated for the simultaneous quantitative determination of 24 β-agonists in human urine proved adequate to measure the real concentration of these analytes in real samples of patients with asthma or COPD. The method proved simple, accurate and highly sensitive, allowing the simultaneous detection of all compounds within a short run time. In comparison with the method we previously used [23], more analytes (24 instead of 15) and increased sensitivity (at least one order of magnitude) were gained. The results on real samples allowed us to directly verify that salbutamol and salmeterol tend to accumulate in the body, when the therapy is administered for long periods of time, even if the excretion appears to vary significantly from one patient to another. Moreover, very low concentrations were detected after formoterol assumption, even when the drug was administered in high dosage to control acute asthma episodes.

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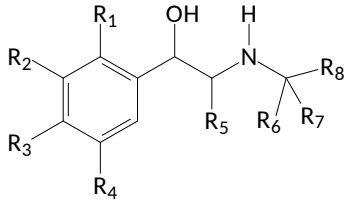
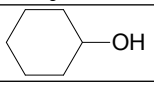
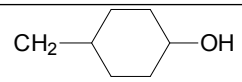
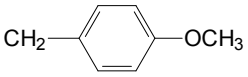
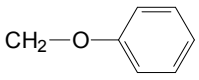
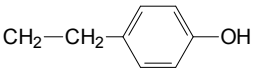
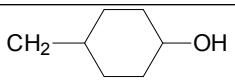
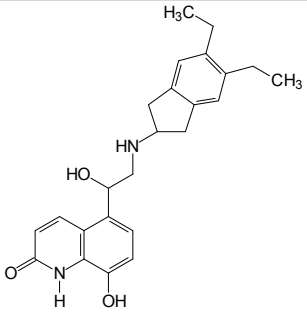
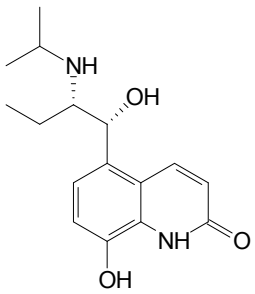
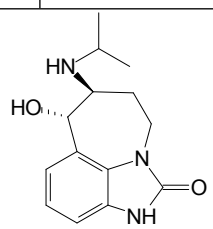
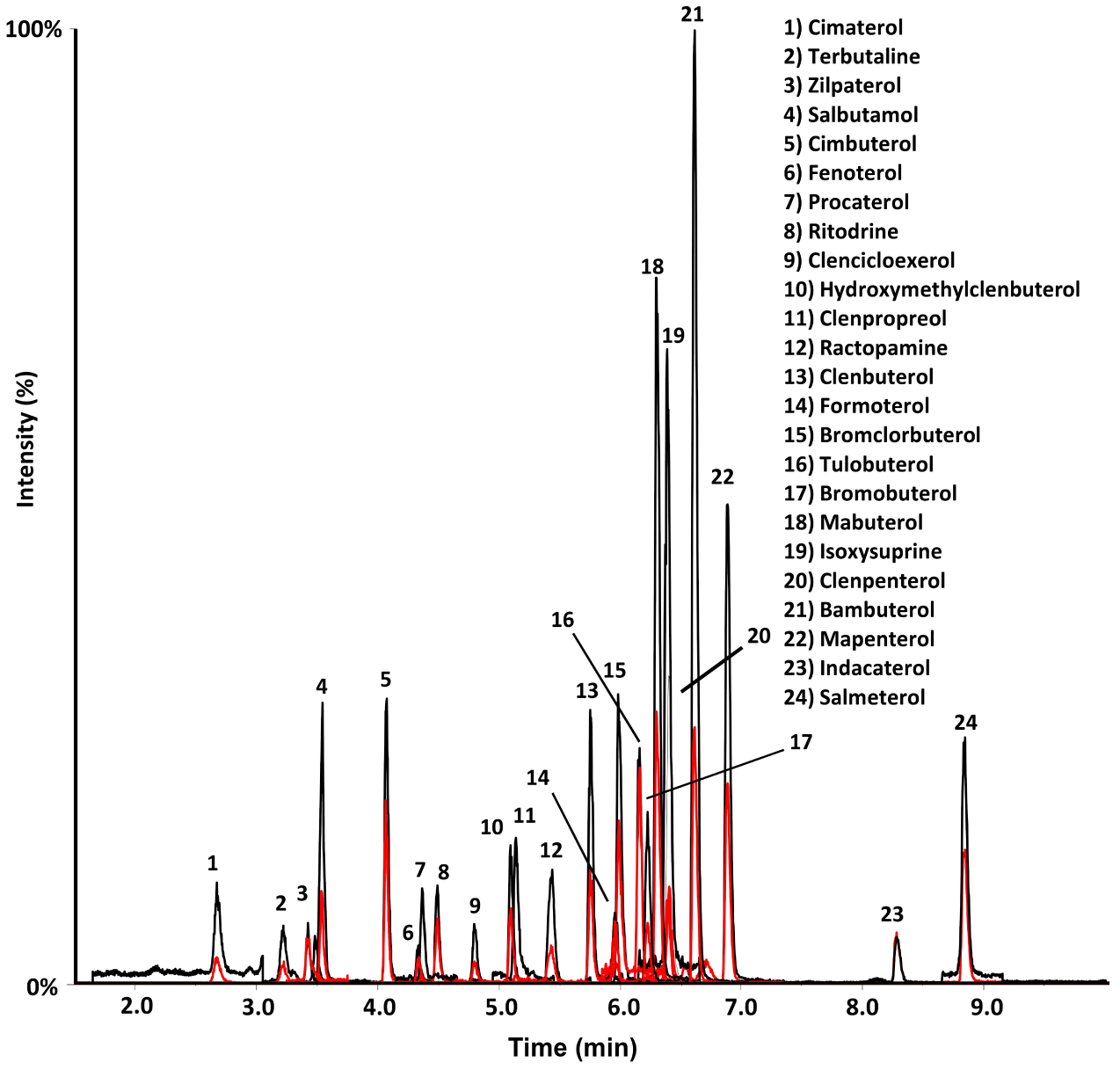


β_2-agonist compounds	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**	**R_7**	**R_8**
Bambuterol	H	$N(CH_3)_2COO$	H	$N(CH_3)_2COO$	H	CH_3	CH_3	CH_3
Bromchlorbuterol	H	Cl	NH_2	Br	H	CH_3	CH_3	CH_3
Bromobuterol	H	Br	NH_2	Br	H	CH_3	CH_3	CH_3
Cimaterol	H	C≡N	NH_2	H	H	H	CH_3	CH_3
Cimbuterol	H	C≡N	NH_2	H	H	CH_3	CH_3	CH_3
Clenbuterol	H	Cl	NH_2	Cl	H	CH_3	CH_3	CH_3
Clencyclohexerol	H	Cl	NH_2	Cl	H	H		
Clenpenterol	H	Cl	NH_2	Cl	H	C_2H_5	CH_3	CH_3
Clenproperol	H	Cl	NH_2	Cl	H	H	CH_3	CH_3
Fenoterol	H	OH	H	OH	H	CH_3		H
Formoterol	H	NH-COH	OH	H	H	CH_3		H
Hydroxymethylclenbuterol	H	Cl	NH_2	Cl	H	CH_2OH	CH_3	CH_3
Isoxsuprine	H	H	OH	H	CH_3	CH_3		H
Mabuterol	H	CF_3	NH_2	Cl	H	CH_3	CH_3	CH_3
Mapenterol	H	CF_3	NH_2	Cl	H	C_2H_5	CH_3	CH_3
Ractopamine	H	H	OH	H	H	CH_3		H
Ritodrine	H	H	OH	H	CH_3	H		H
Salbutamol	H	CH_2OH	OH	H	H	CH_3	CH_3	CH_3
Salmeterol	H	CH_2OH	OH	H	H	H	$-(CH_2)_6O(CH_2)_4Ph$	H
Terbutaline	H	OH	H	OH	H	CH_3	CH_3	CH_3
Tulobuterol	Cl	H	H	H	H	CH_3	CH_3	CH_3
Indacaterol						Procaterol		
Zilpaterol								


100%
0%
Intensity (%)
Time (min)
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
1) Cimaterol
2) Terbutaline
3) Zilpaterol
4) Salbutamol
5) Cimbuterol
6) Fenoterol
7) Procaterol
8) Ritodrine
9) Clencicloexerol
10) Hydroxymethylclenbuterol
11) Clenpropreol
12) Ractopamine
13) Clenbuterol
14) Formoterol
15) Bromclorbuterol
16) Tulobuterol
17) Bromobuterol
18) Mabuterol
19) Isoxysuprine
20) Clenpenterol
21) Bambuterol
22) Mapenterol
23) Indacaterol
24) Salmeterol

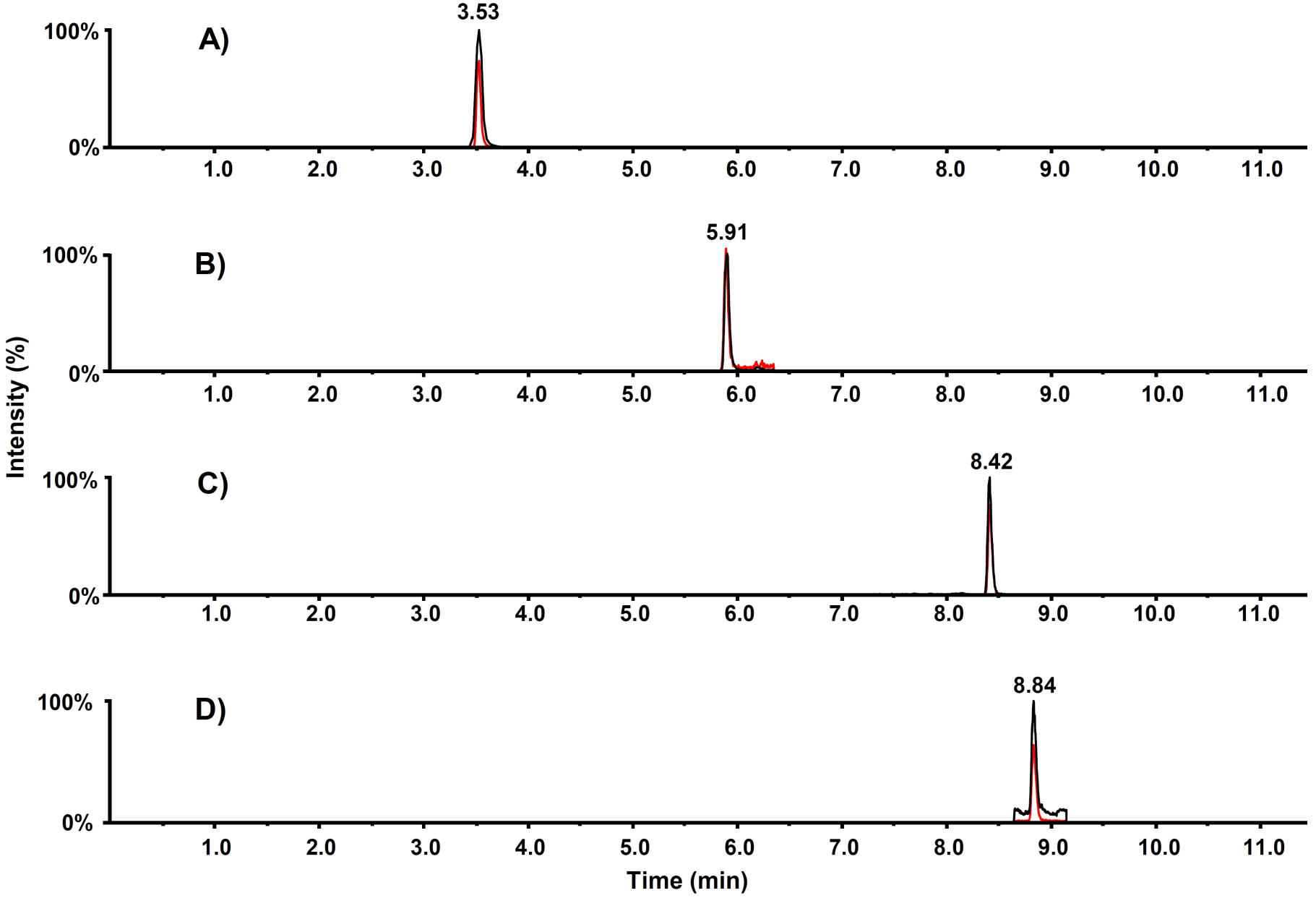

A)
3.53
B)
5.91
C)
8.42
D)
8.84
100%
0%
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
Intensity (%)
Time (min)

Figure captions.

Figure 1. Chemical structures of β_2-agonists considered in this study.

Figure 2. SRM chromatograms of a blank urine sample spiked at 0.500 ng/mL for all the analytes. In black is reported the quantifier fragment and in red the qualifier fragment.

Figure 3. SRM chromatogram of four real samples positive to A) salbutamol at 171±21 ng/mL (patient 003), B) formoterol at ~~0.65~~0.646±0.090 ng/mL (patient 002), C) indacaterol at 3.93±0.32 ng/mL (patient 006) and D) salmeterol at 0.374±0.048 ng/mL (patient 010). In black is reported the quantifier fragment and in red the qualifier fragment.

Table 1. SRM transitions and corresponding potentials for the target compounds and internal standards detection.

Analyte	t_R (min)	Precursor Ion	DP (V)	EP (V)	~~Target~~ Quantifier fragment	CE (V)	CXP (V)	Qualifier fragment	CE (V)	CXP (V)
Cimaterol[b]	2.70	219.9	33	10	202.1	14	15	143.3	32	15
Terbutaline[a]	3.23	226.2	30	7	152.1	27	17	125.2	36	12
Zilpaterol[a]	3.43	262.1	28	10	185.2	30	9	244.3	16	21
Salbutamol[a]	3.54	240.2	30	7	148.0	25	7	166.1	18	7
Cimbuterol[b]	4.06	234.2	43	7	160.0	21	7	143.0	35	10
Fenoterol[a]	4.33	304.3	100	6	135.1	27	10	107.2	49	8
Procaterol[b]	4.36	291.2	49	10	273.2	21	25	214.1	33	20
Ritodrine[a]	4.49	288.1	50	8	150.0	26	10	121.1	29	12
Clencicloexerol[b]	4.79	319.0	42	10	203.0	29	8	132.1	45	8
Hydroxymethylclenbuterol[b]	5.09	293.0	40	10	203.2	26	13	132.0	41	13
Clenproperol[b]	5.14	263.0	45	8	203.1	27	8	188.1	27	8
Ractopamine[a]	5.43	302.2	40	11	164.1	24	14	136.1	33	10
Clenbuterol[b]	5.76	277.0	40	10	203.1	27	20	132.1	42	16
Formoterol[c]	5.95	345.2	25	9	149.0	25	10	121.1	48	11
Bromchlorbuterol[b]	5.98	321.0	33	10	246.9	25	10	302.6	26	10
Tulobuterol[b]	6.15	228.1	50	10	117.9	33	8	154.0	26	11
Bromobuterol[b]	6.23	367.0	30	10	293.1	25	13	212.2	42	13
Mabuterol[b]	6.30	311.1	35	10	236.9	23	13	217.1	36	13
Isoxsuprine[a]	6.39	302.1	42	10	284.1	20	15	150.3	31	15
Clenpenterol[b]	6.39	290.9	43	10	203.0	22	15	132.1	38	15
Bambuterol[a]	6.61	368.2	43	10	294.0	28	8	212.2	20	10
Mapenterol[b]	6.88	325.0	40	10	237.2	22	15	217.2	36	15
Indacaterol[a]	8.27	393.2	64	10	173.1	33	10	117.0	63	10
Salmeterol[d]	8.84	416.3	75	9	398.2	22	11	380.2	27	11
Salbutamol D3[a]	3.52	243.2	38	10	151.0	26	15	-	-	-
Clenbuterol D6[b]	5.73	283.0	40	10	203.0	18	15	-	-	-
Formoterol ^{13}C D3[c]	5.92	349.1	32	10	153.1	27	9	-	-	-
Salmeterol D3[d]	8.83	419.2	83	12	401.2	22	11	-	-	-
DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Cell Exit Potential										

Table 2. Calibration interval, squared correlation coefficient, LODs and LOQs values, Lack of Fit's, ANOVA, Mandel's, RDS slope and Back calculation test results for all analytes.

Analyte	Experimentally tested Linearity range (ng/mL)	Correlation coefficient (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Lack of fit's test (F_{exp})	ANOVA (F_{exp})	Mandel's test (F_{exp})	RSD slope test (%)	Back calculation test (%)
Cimaterol	0.15-5.0	0.9983	0.0329	0.0586	0.24	0.494	1.34	2.98	6.07
Terbutaline	0.15-5.0	0.9969	0.0439	0.0878	0.67	0.707	1.47	2.85	10.1
Zilpaterol	0.15-5.0	0.9989	0.023	0.0546	0.16	0.415	1.23	2.63	3.90
Salbutamol	0.15-5.0	0.9998	0.011	0.022	0.04	0.285	1.45	2.00	1.88
Cimbuterol	0.03-2.5	0.9994	0.0109	0.0218	0.07	0.502	1.31	3.35	5.73
Fenoterol	0.15-5.0	0.9959	0.045	0.089	0.34	0.673	1.85	4.46	7.03
Procaterol	0.15-5.0	0.9994	0.0218	0.04035	0.08	0.664	1.40	3.72	5.25
Ritodrine	0.15-5.0	0.9955	0.0547	0.094	0.58	0.467	2.48	3.03	13.3
Clencicloexerol	0.15-5.0	0.9999	0.0071	0.014	0.03	0.127	1.04	1.11	2.84
Hydroxymethylclenbuterol	0.15-5.0	0.9999	0.0071	0.014	0.04	0.302	1.26	1.47	1.83
Clenproperol	0.15-5.0	0.9993	0.0218	0.0364	0.16	0.376	2.31	1.98	4.37
Ractopamine	0.15-5.0	0.9995	0.0216	0.031	0.07	0.794	1.30	3.89	1.89
Clenbuterol	0.15-5.0	0.9990	0.022	0.044	0.10	0.926	1.32	4.87	5.18
Formoterol	0.15-5.0	0.9998	0.010	0.021	0.06	0.081	1.09	0.84	2.38
Bromchlorbuterol	0.15-5.0	0.9993	0.0182	0.00436	0.09	0.510	1.59	3.01	5.47
Tulobuterol	0.03-2.5	0.9990	0.011	0.022	0.14	0.593	1.20	3.23	6.73
Bromobuterol	0.03-2.5	0.9979	0.0216	0.032	0.34	0.886	1.02	3.69	11.4
Mabuterol	0.15-5.0	0.9998	0.011	0.021	0.03	0.474	1.00	3.17	2.45
Isoxsuprine	0.15-5.0	0.9990	0.022	0.043	0.16	0.334	1.99	2.26	3.26
Clenpenterol	0.15-5.0	0.9990	0.022	0.045	0.16	0.443	1.97	2.64	3.99
Bambuterol	0.03-2.5	0.9992	0.010	0.020	0.18	0.373	2.17	1.99	6.46
Mapenterol	0.03-2.5	0.9977	0.0217	0.034	0.34	0.465	2.00	2.80	9.56
Indacaterol	0.15-5.0	0.9990	0.023	0.0465	0.18	0.572	1.73	2.87	2.85
Salmeterol	0.03-2.5	0.9998	0.0051	0.010	0.05	0.235	1.61	1.58	4.88

[a]Calibration levels (ng/mL) = 0.15 – 0.25 – 0.5 – 1.0 – 2.5 – 5.0

[b]Calibration levels (ng/mL) = 0.03 – 0.1 – 0.25 – 0.5 – 1.0 – 2.5

Lack of fit's test – F_{crit} = 2.776 (n_1 = 4 and n_2 = 24 degrees of freedom)
ANOVA – F_{tab} = 3.842 (n_1 = 1 and n_2 = 28 degrees of freedom)
Mandel's test - F_{crit} = 2.62 (n_1 = 5 and n_2 = 24 degrees of freedom)
RSD slope test - %RSD threshold = 5.00%

Back calculation test - % threshold = 20%

Table 3. Intra-day precision (CV%), accuracy (bias%), matrix effect and extraction recovery for all the analytes tested.

	Level I			Level II			Level III			Matrix effect (%)	Recovery (%)		
Analyte	**Conc. (ng/mL)**	**Precision (CV%)**	**Accuracy (bias%)**	**Conc. (ng/mL)**	**Precision (CV%)**	**Accuracy (bias%)**	**Conc. (ng/mL)**	**Precision (CV%)**	**Accuracy (bias%)**	**Level I**	**Level I**	**Level II**	**Level III**
Cimaterol	0.15	16	+5.5	0.50	9.7	+0.1	2.5	5.1	+1.5	-33	108	100	101
Terbutaline	0.15	12	+1.3	0.50	7.3	-1.2	2.5	2.5	+1.6	+0.05	97	100	99
Zilpaterol	0.15	9.4	+1.2	0.50	8.3	+1.6	2.5	4.8	+2.0	-1.7	107	110	96
Salbutamol	0.15	7.1	-4.7	0.50	4.5	-1.4	2.5	4.0	+2.3	+5.7	91	94	100
Cimbuterol	0.03	7.5	+6.7	0.25	4.5	+3.5	1.0	4.5	+2.4	+5.7	97	95	97
Fenoterol	0.15	12	+8.4	0.50	5.2	-1.8	2.5	3.7	+2.4	-2.7	116	103	103
Procaterol	0.15	13	+3.2	0.50	3.7	-3.0	2.5	1.1	+0.4	+4.2	98	88	102
Ritodrine	0.15	19	+7.4	0.50	4.8	-0.9	2.5	3.4	+0.6	-19.3	95	102	97
Clencicloexerol	0.15	5.0	+4.6	0.50	4.3	+0.6	2.5	3.3	+0.3	-8.0	113	111	95
Hydroxymethylclenbuterol	0.15	11	-0.9	0.50	3.4	-3.1	2.5	0.3	+0.02	-1.9	98	107	96
Clenproperol	0.15	11	-0.7	0.50	7.0	+2.6	2.5	0.8	+0.2	+12	89	100	96
Ractopamine	0.15	16	+15	0.50	8.8	+1.8	2.5	1.6	-0.6	-1.2	106	98	100
Clenbuterol	0.15	9.9	+9.8	0.50	8.4	+1.0	2.5	7.1	+1.8	+23	98	99	98
Formoterol	0.15	10	+2.8	0.50	6.0	+1.1	2.5	2.4	-0.5	+17	102	105	98
Bromchlorbuterol	0.15	8.0	-0.2	0.50	1.5	+0.3	2.5	2.8	+0.8	+2.6	99	85	100
Tulobuterol	0.03	11	+10	0.25	3.2	-1.0	1.0	5.0	-0.4	+9.2	110	89	98
Bromobuterol	0.03	2.3	+3.4	0.25	3.4	+0.3	1.0	2.6	+0.5	-3.7	102	92	105
Mabuterol	0.15	10	+14	0.50	7.2	+0.1	2.5	7.3	-2.5	+16	101	102	99
Isoxsuprine	0.15	11	+1.8	0.50	2.5	-0.4	2.5	0.6	-0.1	-2.5	99	96	101
Clenpenterol	0.15	1.9	+0.4	0.50	3.8	-0.6	2.5	3.2	+1.3	-4.2	102	96	98
Bambuterol	0.03	3.5	+6.9	0.25	4.7	+3.2	1.0	2.1	+0.5	+5.0	104	96	98
Mapenterol	0.03	17	+13	0.25	2.8	+2.1	1.0	2.8	+0.3	+8.7	80	94	105
Indacaterol	0.15	13	+4.5	0.50	5.3	+0.8	2.5	1.6	+0.4	+1.2	100	110	106
Salmeterol	0.03	18	-0.4	0.25	2.9	+3.7	1.0	0.5	-0.4	-3.8	110	88	99

Table 4. Patients under chronic therapy. General features, treatment information, and β-agonists concentration determined in urine.

CHRONIC THERAPY							
β-agonist	**Patient**	**Sex**	**Age**	**Time in therapy (months)**	**Time from last administration (h)**	**Dose (µg)**	**Urine concentration (ng/mL)**
Salmeterol	010	F	50	36	1	25+25	0.37
	012	F	72	2	3	50+50	0.02
	039	F	64	6	3	50+50	0.08
	043	F	65	36	3	50+50	0.38
	044	F	39	3	3	25+25	0.07
	050	M	56	60	3	25+25	0.55
	017	F	64	10	5	50+50	0.22
	035	M	43	4	5	50+50	0.11
	058	F	28	2	6	50+50	ND
	022	M	81	3	7,5	50+50	0.16
	041	F	59	72	8	50+50	0.16
	040	M	38	24	48	50+50	ND
Indacaterol	006	F	66	0.5	24	150	3.93
Formoterol	054	F	30	2	0.5	12+12	4.05
	004	F	60	1	1	6+6	1.20
	016	F	83	0.25	1.5	6	0.50
	046	F	27	120	2	12+12	41.2
	029	M	53	15	3	12+12	1.78
	031	F		3	3	6+6	1.99
	037	M	23	60	3	4,5+4,5	0.81
	057	F	47	3	3	12+12	1.16
	020	F	46	48	4	12+12	0.14
	030	F	48	0.25	4	12+6	15.9
	056	M	20	1	4	4,5+4,5	0.63
	014	F	34	3	5	6+6	0.77
	019	F	49	1	5	4,5 + 4,5	0.30
	028	M	54	2.5	5	9	2.00
	005	M	75	0.25	6	12+12+12	0.62
	032	F	45	2	6	6+6	0.46
	038	F	49	60	6	4,5 + 4,5	2.69
	055	F	54	36	7	6+6	2.20
	011	M	56	72	8	4,5	0.23
	042	F	65	132	8	4,5+4,5	0.30
	021	F	70	5	14	6+6	1.20
	023	F	26	1.5	14	6+6	0.05
	013	F	30	2	14	12	0.14
	033	M	16	10	15	12+12	0.88
	045	M	29	8	15	6+6	ND
	018	F	54	36	17	6+6	ND
	034	F	41	12	17	4,5 + 4,5	0.10
	025	F	43	0.75	20	12+12	0.12
	048	F	34	12	30	6	1.88
	047	F	62	1.5	48	6	ND
	009	F	13	1	52	6+6	< LOQ
Salbutamol	059	M	38	12	3	200	57
	006	F	66	16 years	18 days	50+50	42.1

*ND = not detected

Table 5. Patients under acute therapy. General features, treatment information, and β-agonists concentration determined in urine.

ACUTE THERAPY						
β-agonist	**Patient**	**Sex**	**Age**	**Time in therapy (h)**	**Dose (µg)**	**Urine concentration (ng/mL)**
Formoterol	015	M	30	0.25	4.5	<LOQ
	007	F	38	0.5	5+5	2.0
	002	M	61	4	200	0.65
	053	F	52	6	6	0.41
	027	M	33	8	18	1.3
	049	M	29	16	9	ND
	001	F	33	17.5	4.5	<LOQ
	003	M	33	18.5	4.5	<LOQ
	036	F	24	48	4.5+4.5	0.09
Salbutamol	051	F	48	0.5	400	171
	052	F	53	1	400	926
	060	M	34	1	400	103
	003	M	33	1.5	300	171
	008	F	70	2	200	1.20
	013	F	30	2	400	138
	024	M	21	2	400	406
	040	M	38	3	400	295
	027	M	33	12	1312	41.4
	026	F	29	15	200	12.8

* ND = not detected